Transcriptional response of the heat shock gene hsp70 aligns with differences in stress susceptibility of shallow-water corals from the Mediterranean Sea

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ABSTRACT

Shallow-water corals of the Mediterranean Sea are facing a dramatic increase in water temperature due to climate change, predicted to increase the frequency of bleaching and mass mortality events. However, supposedly not all corals are affected equally, as they show differences in stress susceptibility, as suggested by physiological outputs of corals along temperature gradients and under controlled conditions in terms of reproduction, demography, growth, calcification, and photosynthetic efficiency. In this study, gene expression and induction of a 70-kDa heat shock protein (HSP70) was analyzed in five common shallow-water hard corals in the Mediterranean Sea, namely *Astroides calycularis*, *Balanophyllia europaea*, *Caryophyllia inornata*, *Cladocora caespitosa*, and *Leptopsammia pruvoti*. The main aim was to assess the contribution of this evolutionary conserved cytoprotective mechanism to the physiological plasticity of these species that possess different growth modes (solitary *vs* colonial) and trophic strategies (zooxanthellate *vs* azooxanthellate). Using quantitative real-time PCR, *in situ hsp70* baseline levels and expression profiles after a heat-shock exposure were assessed.

Levels of *hsp70* and heat stress induction were higher in zooxanthellate than in azooxanthellate species, and different heat stress transcriptional profiles were observed between colonial and solitary zooxanthellate corals. On the whole, the *hsp70* transcriptional response to heat stress aligns with stress susceptibility of the species and suggests a contribution of trophic strategy and morphology in shaping coral resilience to stress. Understanding these molecular processes may contribute to assess the potential effects and relative resilience of Mediterranean corals under climate change.

1. Introduction

Marine ecosystems are declining worldwide due to global climate change and other anthropogenic impacts (Hughes et al., 2018). These rapid changes alter the structure of habitats and communities and cause the loss of many species. Of particular concern is the decline of scleractinian corals triggered by thermal stress (Hoegh-Guldberg et al., 2007). Rising sea surface temperature is linked to coral bleaching and mass mortality events in both symbiotic and non-symbiotic corals in the tropics and in the Mediterranean Sea (Altieri et al., 2017; Jimenez et al., 2016; Rodolfo-Metalpa et al., 2006b). The Mediterranean Sea is warming at two to three times the rate for the global ocean (Vargas-Yáñez et al., 2008), showing an increased occurrence of hot extremes by 200–500% throughout the region (Diffenbaugh et al., 2007). This is due to its position in the transition between the arid climate of North Africa and the temperate climate of central Europe, which renders Mediterranean climate vulnerable to even relatively moderate modifications of the general circulation (Giorgi and Lionello, 2008). Furthermore, its

semi-enclosed nature and the restricted water exchanges with the Atlantic Ocean results in a hydrological residence time of about 100 years and a great capacity to store heat (Diffenbaugh et al., 2007). Indeed, climatic models predict a rapid average warming in the Mediterranean region (about 2.5 °C over the 21st century under the RCP8.5 projection; Shaltout and Omstedt (2014)) along with a greater occurrence of extreme temperature events (Giorgi and Lionello, 2008). Under such a scenario, not all corals are thought to be affected equally: during mass coral bleaching events, survival of scattered colonies suggests that some groups of corals may possess inherent physiological tolerance to environmental stress (Marshall and Baird, 2000; Oliver and Palumbi, 2011; West and Salm, 2003). Therefore, questions remain about the underlying mechanisms leading to differences in thermal tolerance, and about the relative contributions of genotypic adaptation (where selection drives differences in susceptibility over evolutionary times scales), and phenotypic acclimatization (where organisms respond to extremes using their existing genomic repertoire) (Palumbi et al., 2014).

Changing environmental conditions may be physiologically tolerable, allowing acclimatization or adaptation, or may be intolerable, promoting change in phenology (timing of annual events), or massive bleaching, death and local extinction (Parmesan, 2006). Physiological performances and responses are the principal determinant of a species tolerance to environmental variability and, as such, they may change the ability of individual corals to cope with changing conditions (Somero, 2012). Physiological plasticity describes the process of tuning phenotypic traits of organisms within their lifetime allowing them to live in varying environments (Gates and Edmunds, 1999). In this regard, investigations of the regulatory mechanisms governing stress responses by monitoring changes of mRNA expression profiles may provide early-warning molecular markers of physiological plasticity and represent an important source of information on the mechanisms underlying the differences in coral physiological resilience (Dixon et al., 2015; Poli et al., 2017; Seneca and Palumbi, 2015).

Although studies on tropical corals disclosed some molecular mechanisms underlying acclimatization and coordinated modulation of gene expression as a reaction to environmental stress (Bay and Palumbi, 2017; Bellantuono et al., 2012; Gutner-Hoch et al., 2017; Maor-Landaw et al., 2014), similar information on temperate corals of the Mediterranean Sea are largely lacking. To fill this gap, the present study assessed expression of a *hsp70* gene product under baseline and heat stress conditions in five Mediterranean shallow-water coral species representing different growth modes (colonial: *Astroides calycularis* and *Cladocora caespitosa*; solitary: *Balanophyllia europaea*, *Caryophyllia inornata,* and *Leptopsammia pruvoti*) and trophic strategies (zooxanthellate: *B. europaea* and *C. caespitosa*; azooxanthellate: *A. calycularis*, *C. inornata,* and *L. pruvoti*) (Fig. 1). An overview of population dynamics and physiological traits (i.e., reproduction, linear extension rate, net calcification, and photosynthetic efficiency) of these species in relation to increasing temperature is provided in Fig. 1, as a means to infer their relative tolerances to stress. For example, azooxanthellate species *C. inornata* and *L. pruvoti* seem quite tolerant to temperature increases compared to their sympatric zooxanthellate species *B. europaea* and *C. caespitosa*, while *A. calycularis* responds negatively to increasing temperatures (Fig. 1).

The 70-kDa heat shock proteins (HSP70s) are molecular chaperones and have vital cytoprotective functions (Feder and Hofmann, 1999). They are involved in protein folding, unfolding, sorting transport, and assembly of complexes. They also protect cells from apoptosis and proteotoxic stressors (Hartl et al., 2011). During exposure to elevated temperatures, protein misfolding, aggregation or disruption of regulation and disassembly of multiprotein complexes may occur, leading to subsequent activation of signaling pathways triggering a stress-related HSP70 induction (Richter et al., 2010). Through their cytoprotective functions, HSP70 are thought to restore proteolytic homeostasis.

HSP70s are broadly studied, highly conserved molecular mediators of environmental acclimatization in marine organisms (Fabbri et al.,

2008; Morris et al., 2013), and amongst the primary early responders to stress in corals (Louis et al., 2017; Traylor-Knowles et al., 2017). As a consequence, many studies assessed hsp70 expression in tropical corals under different abiotic and biotic cues, including temperature (Maor-Landaw et al., 2014; Seveso et al., 2014; Zhang et al., 2018), pH/pCO_2 (Moya et al., 2015), salinity (Ellison et al., 2017), pollutants (Jovanović and Guzmán, 2014; Overmans et al., 2018; Venn et al., 2009), bacterial infections (Brown et al., 2013; Seveso et al., 2016), or host-symbiont interplay to maintain holobiont homeostasis (Levy et al., 2011; Rosic et al., 2014b). These studies suggest that cnidarian hsp70 gene expression and function is similar across multiple stressors and comparable to vertebrates (Kvitt et al., 2016). Furthermore, *hsp70* up-regulation at the initial stage of the stress response appears to be a common protective mechanism to avoid the onset of severe pathological conditions or to postpone bleaching (Maor-Landaw et al., 2014; Rosic et al., 2014a).

Studies on hsp70 expression in relation to environmental variables or to symbiosis in Mediterranean corals are lacking, and hsp70 sequences are not available for the species investigated in this study. An exception is represented by *B. europaea*, for which a recent investigation based on whole transcriptome sequencing reported *hsp70* transcriptional response to temperature and pH combined exposures (Maor-Landaw et al., 2017). The comparison with the tropical *Stylophora pistillata* suggested that *B. europaea* is endowed with more efficient protective and metabolic processes to cope with the high thermal variability of Mediterranean environments (Maor-Landaw et al., 2017). To our knowledge, this is the first study reporting a comparative multispecies analysis of the HSP70 response to thermal stress in temperate corals, which attempts to set the basis for future investigations on the potential regulatory mechanisms behind physiological resilience and acclimatization capabilities in corals from the Mediterranean Sea. We investigated transcriptional regulation of a stress inducible *hsp7*0 by concomitant analysis of expression levels under field conditions and following short-term heat stress experiments, to infer possible influences of peculiar physiological features and diverse life history traits. Gaining this knowledge is a first important step towards understanding the processes involved in species resilience to heat-stress events and may disclose the relevant threats involved in their decline.

2. Methods

2.1. Coral sampling and experimental design

During May 2016, 60 nubbins (from 6 colonies) of *C. caespitosa* and 60 polyps of *B. europaea*, *C. inornata,* and *L. pruvoti* were collected by SCUBA diving at Calafuria (Leghorn; 43°28.4′N, 10°20′E) (Fig. 2). Nubbins (60 from 6 colonies) of *A. calycularis* were collected at Ponza Island (40°54′N 12°58′E) (Fig. 2). Samples were collected from sites characterized by high population density at 5–30 m depth, and all samplings were performed at the same time of the day (early morning), to minimize possible biases due to Hsp70 diel modulation (Levy et al., 2011; Seveso et al., 2018). Upon collection, 12 randomly selected single polyps or fragments of colonies (2 nubbins *per* colony) from each species were immediately immersed in a suitable volume of the RNA later stabilization reagent (Sigma-Aldrich, Milan, Italy) and frozen at - 80 °C for further analysis of transcriptional levels of coral *hsp70* under *in situ* baseline conditions. The remaining samples were immediately put in refrigerated seawater and transported to the aquarium system of the Department of Biological, Geological and Environmental Sciences (BiGeA) of the University of Bologna. They were acclimatize for 40 days at a constant water temperature of 17 °C ± 0.5 °C matching the average temperature at the collection sites (Fig. 2B and C). Zooxanthellate species (*B. europaea* and *C. caespitosa*) were maintained at a photoperiod of 15 h:9 h light:dark daily cycles to match the natural photoperiod at the collection sites. Azooxanthellate species (*A. calycularis*, *C. inornata,* and *L. pruvoti*) were maintained under dimmed light
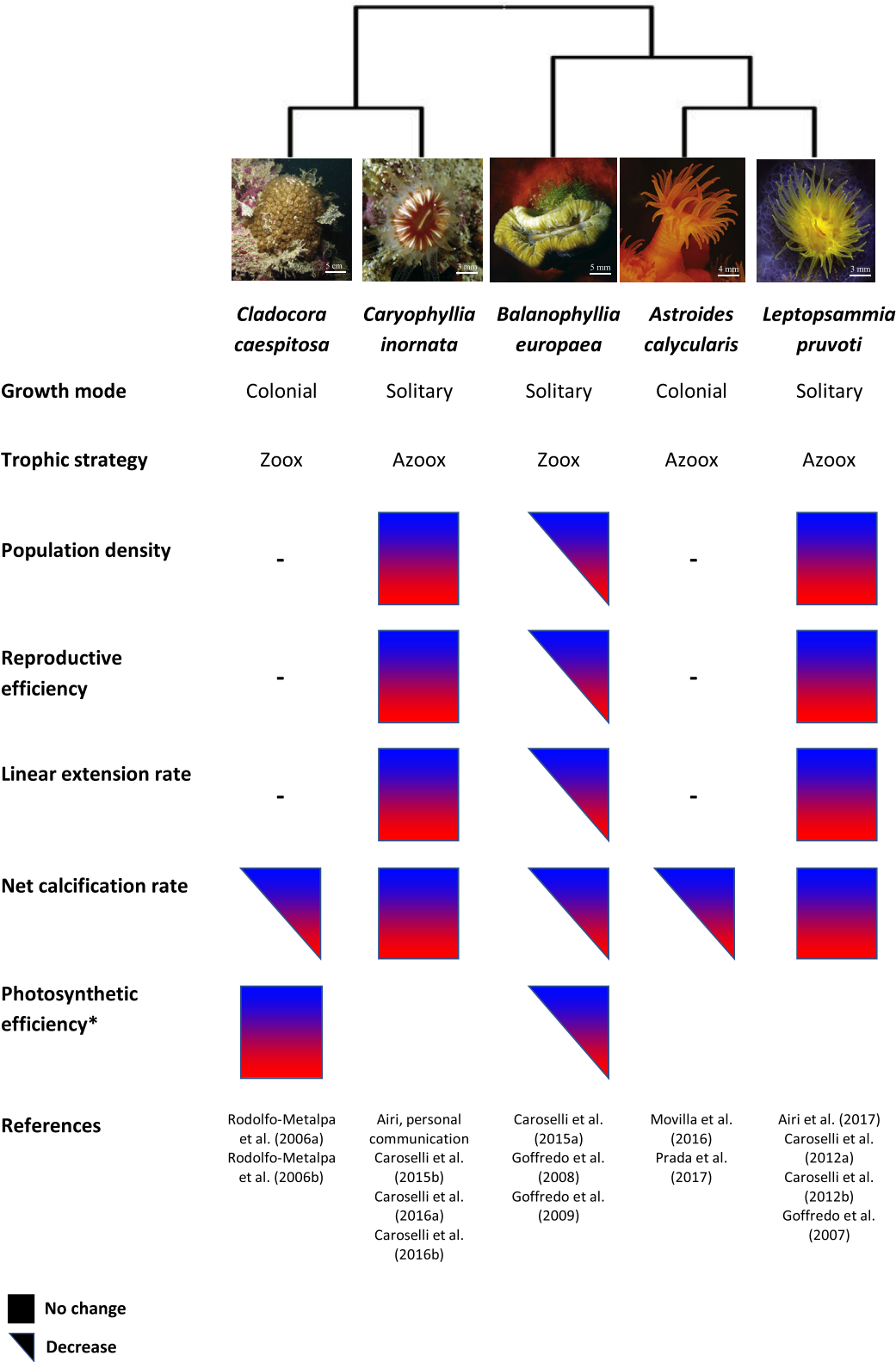
Fig. 1. Mediterranean shallow-water corals used in this study alongside phylogenetic relationships and physiological outputs in relation to temperature. Data on population density, reproductive efficiency, linear extension rate, and net calcification refer to field studies conducted along temperature gradients, and photosynthetic efficiency refers to experiments under controlled conditions. Shapes indicate the species temperature-related response (square: no change; triangle: decrease), colors indicate the direction of temperature change reported in the referred papers, from lower (blue) to higher (red) temperatures. Zoox: zooxanthellate. Azoox: azooxanthellate. - no data available. Coral pictures are from Francesco Sesso (Caroselli et al., 2015a; Caroselli et al., 2015b; Caroselli et al., 2012b; Goffredo et al., 2009; Goffredo et al., 2008) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

conditions to mimic their natural environment. During the acclimatization period, corals were fed twice a week with a commercial plankton preparation (Elos Coral Foods SvC).

Thermal stress experiments were performed using a short-term heat shock treatment at a relatively high temperature (here 32 °C, about 5 °C above average ambient values) followed by 0–24 h of post-stress recovery at the coral acclimatization temperature (Fig. 3). This exposure scheme and experimental parameters were selected according to our past experience on marine invertebrate investigations on the HSP70 response (Franzellitti and Fabbri, 2005; Piano et al., 2005, 2004). Briefly, this experimental setup was chosen to analyze the different capabilities of the selected species to promptly increase *hsp70* transcription within short-term intense heat stress exposures. It also accounts for the specific regulatory features of stress-inducible Hsp70s,
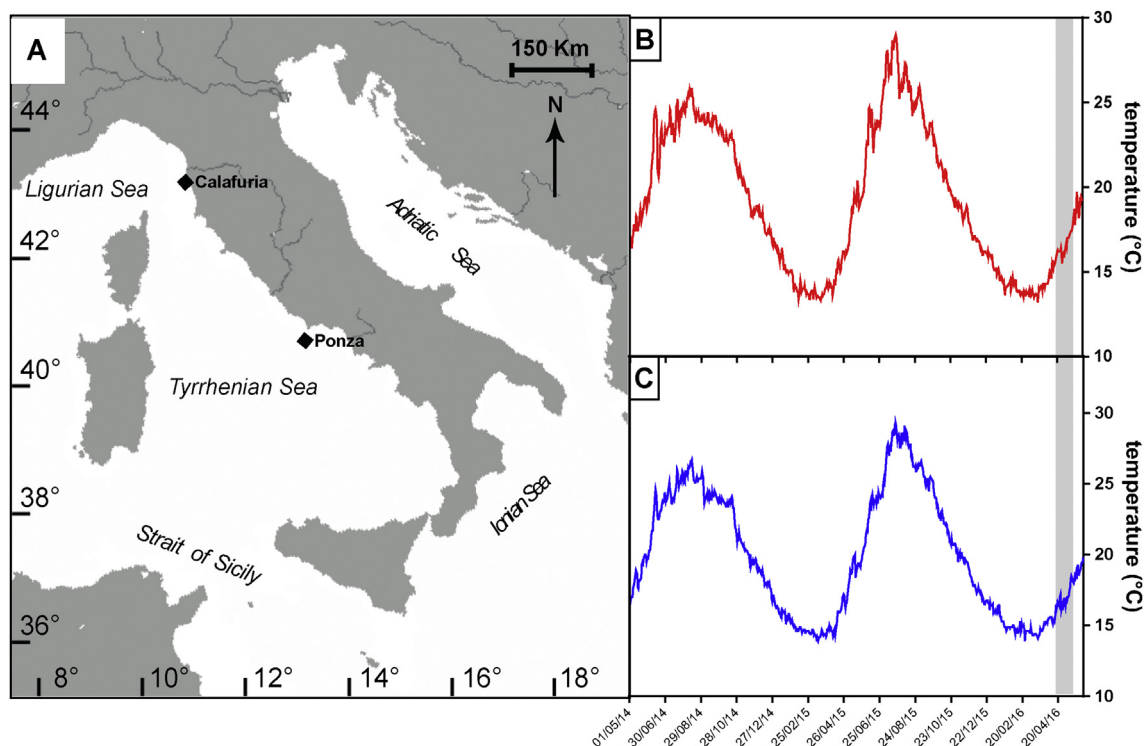
Fig. 2. Map of the sampling sites at Calafuria and Ponza Island (Thyrrenian Sea, Italy). (A) The map of the sampling locations was generated using the OpenStreetMap database (http://www.openstreetmap.org/copyright). Mean satellite-derived daily sea surface temperature (SST) profiles at (B) Calafuria and (C) Ponza sampling locations (across a 2-year period spanning the sampling time point) were retrieved using the Copernicus Marine Service Product (http://marine.copernicus.eu/), and visualized through Panoply ver 4.8 (http://www.giss.nasa.gov/tools/panoply/). Sampling period is showed (grey area). Average SSTs within the sampling period were (mean ± SD) 16.9 ± 0.5 °C (Calafuria) and 17.4 ± 0.7 °C (Ponza) (For interpretation of the references color in this figure legend, the reader is referred to the Web version of this article).

within the earlier phases of the stress response, after which further compensative and/or adaptive processes may be activated (Franzellitti et al., 2010; Franzellitti and Fabbri, 2005; Maor-Landaw et al., 2014; Morris et al., 2013; Rosic et al., 2014a).

After acclimatization, 12 nubbins/polyps for each species were collected to account for differences in animal physiological status at the onset of the experimental exposure to thermal stress, thus representing the control condition of the experiment (2 nubbins/polyps were pooled together to give 6 samples per species; N = 6). The remaining samples were divided in different transparent glass beakers, to facilitate their manipulation during the experiments, and transferred to an experimental tank where the water temperature was raised to 32 °C. The samples were maintained at 32 °C for 2 h, and then allowed to recover for 0-, 2-, or 24-h post-stress periods at the acclimatization temperature (17 °C) (Fig. 3). At each time-point 6 nubbins/polyp per species were

sampled, randomly pooled into 3 samples (2 nubbins/polyps for each sample), immediately immersed in RNA later solution (Sigma Aldrich, Milan, Italy), and stored at −80 °C for subsequent procedures (see below). Thermal stress experiments were settled to allow 0 h and 24 h post-stress recovery samplings at the same time at which the field samplings were performed.

2.2. RNA extraction and cDNA preparation

Nubbin/polyp pools were homogenized in a suitable volume of the TRI Reagent (Sigma Aldrich, Milan, Italy) according to Barshis et al. (2013). Total RNA was further extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) according to the manufacturers' protocol. DNAase I treatment was performed within the RNA extraction procedures according to the manufacturers' instructions (Zymo Research,
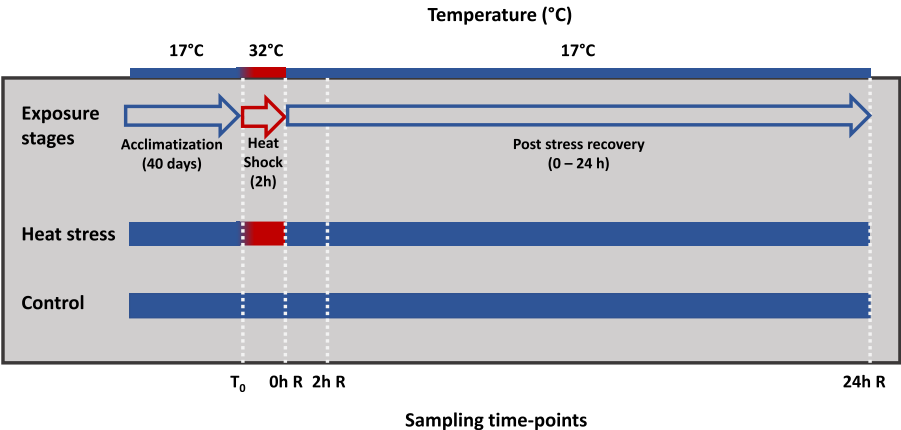

Fig. 3. Scheme of the experimental design for the coral heat shock exposure. T_0: time zero of the heat stress exposure, before the onset of the temperature challenge in the heat stress treatment. Sampling time-points refer to the recovery periods (R) post-stress (For interpretation of the reference color in this figure legend, the reader is referred to the Web version of this article).

Freiburg, Germany). RNA concentration and quality were confirmed using the Qubit system with the Qubit RNA assay kit (Thermo Scientific, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. RNA integrity was evaluated based on the presence of clear 28S and 18S ribosomal RNA bands in the electrophoresis, and by confirming that 28S band intensity was at least double that of 18S. A further qualitative analysis was performed by measuring the UV absorbance spectra of the samples (λ = 200–340 nm). Absorbances (A) at 260, 230, and 280 nm were used to calculate the ratio A_{260}/A_{280} addressing the occurrence of protein contaminations (cut-off values > 1.8 and < 2.0), and the ratio A_{260}/A_{230} addressing the occurrence of contaminants that may be present in the samples, such as guanidine thiocyanate, which is a component of the TRI Reagent (cut-off value > 1.7).

First strand cDNA for each sample was synthesized from 600 ng total RNA using the iScript supermix following the manufacturer's protocol (Bio-Rad Laboratories, Milan, Italy).

2.3. Cloning and sequencing

The cDNAs corresponding to the coral *hsp7*0 mRNAs were amplified by a semi-nested PCR protocol using degenerated primers designed on conserved regions of a multiple alignment of homologous coral protein sequences retrieved from the GenBank and the Reefgenomics.org (Bhattacharya et al., 2016; Liew et al., 2016) databases (Table 1). The multiple alignment was done using the MEGA 6 software (Tamura et al., 2013) and a dataset of coral Hsp70 protein sequences reported by Poli et al. (2017). For each coral species, cDNA employed at this stage were synthetized from pooled heat-stressed coral total RNA samples (2 h at 32 °C + 2 h of post-stress recovery). In the first PCR run, the primer pair CoHSP70F1 (5′-ATHGAYCTNGGNACNACNTA-3′) and CoHSP70R2 (5′-AGNACNSWNACRTCRAANGT-3′) was used. In the second PCR run, products of the first PCR run were used as templates for the reaction with the CoHSP70F1 forward primer and the (internal) reverse primer CoHSP70R1 (5′-NCCNGCRTCYTTNGTNGCYTG-3′). In both runs, PCR reactions were performed in a 25 μL volume containing 3 μL of cDNA template or 1 μL of PCR product, 0.5 μM each primer, 1.5 mM MgCl₂, 0.2 mM each dNTP, and 0.5 units of RedTaq DNA polymerase (Sigma Aldrich, Milan, Italy). The thermal profile consisted of an initial denaturation step (95 °C, 10 min), followed by 38 cycles of denaturation (94 °C, 20 s), annealing (52 °C, 45 s) and extension (72 °C, 2 min), and a final extension step (72 °C, 10 min). PCR products were separated on a 2.5% wide range agarose gel (Sigma Aldrich, Milan, Italy) using TBE buffer (1 M Tris, 0.9 M boric acid, and 1 mM EDTA) and stained with the GelRed staining solution (Biotium, VWR International, Milan, Italy). Gels were analyzed with the Gel Doc™ EZ System and the ImageLab software (Bio-Rad Laboratories, Milan, Italy). PCR products of the expected size were cloned into a pSC-A plasmid vector (Agilent, Milan, Italy) using the StrataClone PCR cloning kit (Agilent, Milan, Italy). No PCR product was detected from negative controls (reactions without reverse transcriptase added). The screening of clones was performed through EcoRI digestion of plasmid DNA, after plasmid extraction with the GenElute plasmid miniprep kit (Sigma Aldrich, Milan, Italy). Sequencing of both strands of the plasmid DNA from positive clones was performed by Sanger sequencing (BMR Genomics, Padoa, Italy). A dataset of available coral hsp70 nucleotide sequences was built using the corresponding protein sequence dataset reported by

Poli et al. (2017). The obtained nucleotide sequences were then aligned and analyzed against this dataset using the MEGA 6 program package (Tamura et al., 2013) to test the degree of sequence identity with other coral hsc70 and hsp70 genes.

2.4. qPCR assays

Levels of *hsp70* transcripts was assayed by quantitative Real Time Polymerase Chain Reaction (qPCR) assays and using a protocol for the absolute quantification of the target transcripts. This approach was chosen to overcome the challenges in identifying stably expressed reference transcripts, which is a demanding issue in relative quantification studies (Bustin et al., 2013). Reliable reference transcripts are reported only for model tropical corals including *Acropora* and *Pocillopora* species (Oshiro et al., 2013; Overmans et al., 2018; Pagarigan and Takabayashi, 2008; Rosic et al., 2014a; van de Water et al., 2015). There is poor or no genomic/transcriptomic data for the species considered in this study, making it difficult to select and statistically validate any choice of best-performing reference transcripts (Balbi et al., 2016; Franzellitti et al., 2015; Rosic et al., 2014a).

A universal hsp70 primer pair was designed with the Primer Express software (Life Technologies, Milan, Italy) using nucleotide sequences obtained in this study (Table 1) and exhibiting low sequence homology to hsp70 from *Symbiodinium* clade A and C to avoid non-target amplification. qPCR standards for the target transcript were prepared by serial dilution of the linearized plasmid DNAs containing the specific transcripts to obtain a standard curve of C_T values *vs* the logarithmic DNA amount. R^2 values for all standard curves were > 0.99. Absolute mRNA abundance was calculated from the standard curves and plotted as copy number ng^{-1} RNA (mean ± SEM). Reactions were performed in a final volume of 10 μL containing 5 μL iTaq Universal SYBR Green Supermix with ROX (Bio-Rad Laboratories, Milan, Italy), 2 μL diluted cDNA or plasmid DNA, and 0.2 μM specific primers. A control lacking the DNA template (no-template) and a minus-reverse transcriptase (no-RT) control were included in the qPCR analysis to ensure the specificity of the amplification.

Technical replicates were performed both within each run/plate (samples in duplicate, qPCR standards in triplicate) and between different runs/plates (standards and samples replicated on different plates). Equal loadings within each qPCR reaction were ensured by checking the amounts of each standard and cDNA sample using the Qubit system with Qubit® dsDNA HS (High Sensitivity) assay kit (Thermo Scientific, Milan, Italy).

Amplification was detected with a StepOne real time PCR system (Life Technologies, Milan, Italy) using a standard "fast mode" thermal protocol. For each target mRNA, melting curves, gel pictures, and sequences of PCR products were analyzed to verify the specificity of the amplified products. PCR products for each species were cloned and sequenced. About 10 positive clones for each PCR product were sequenced that confirmed that they were identical to the respective nucleotide sequences originally employed for designing the selected primer pairs (*L. pruvoti* sequences showed a mean 80% homology with sequences from other 4 selected species). Although potential off-target amplification of *Symbiodinium* transcripts cannot be completely ruled out, the low sequence homology to *Symbiodnium* hsp70 of the employed primer pair in combination with the melting curve analyses confirmed that the employed qPCRs mostly account for changes in mRNA

Table 1
Parameters of primers employed in the real-time qPCR assays.

Primer name	Sequence 5′-3′	Amplicon size (bp)	Tm (°C)	Amplification efficiency[a] (%)
MC_HSP70F	CTTTCCTTCCGAGATAGTCTTCAG	107	62	Samples: 109.8 ± 10.9
MC_HSP70R	TCGCGTACAGGTTGGTAAAG		62	Standards: 108.3 ± 9.6

[a] Amplification efficiencies for samples and standards are reported as mean ± SD. Tm: melting temperature.
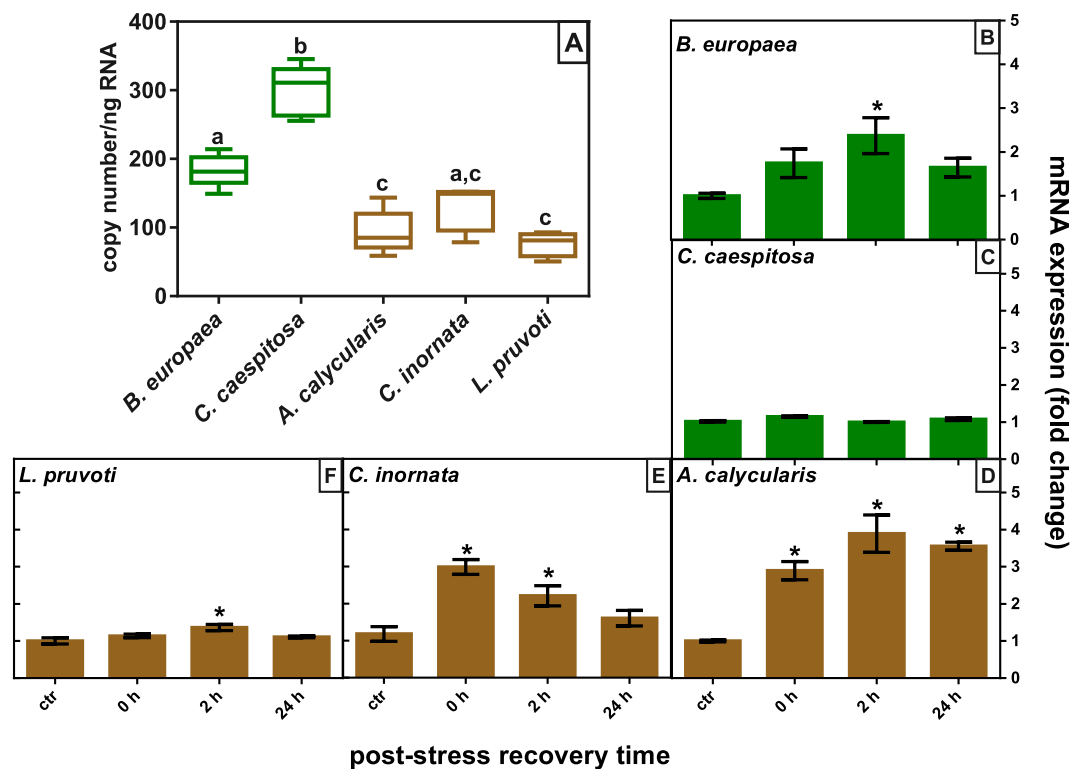
Fig. 4. Expression of *hsp70* in Mediterranean shallow-water corals. (A) *In situ hsp70* baseline expression in *B. europaea*, *A. calycularis*, *C. inornata*, *C. caespitosa*, and *L. pruvoti*. Box-and-whisker plots represent median, upper and lower quartiles (N = 6) of *hsp70* copy number normalized over nanograms of total RNA employed in each PCR reaction. Different letters indicate statistical differences (P < 0.05, Mann-Whytney *U* test). (B–F) Expression of *hsp70* following an acute heat shock treatment (2 h at 32 °C) and several post-stress recovery periods (0–24 h) at 17 °C. Levels of *hsp70* are expressed as the relative variation (fold change) to controls (means ± SEMs; N = 3). *P < 0.05 vs ctr (Mann-Whytney *U* test). Species in green = *B. europaea* and *C. caespitosa* (zooxanthellate); species in brown = *A. calycularis*, *C. inornata*, and *L. pruvoti* (azooxanthellate) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

expression profiles of the coral species.

The amplification efficiency was calculated using a dilution series of samples cDNAs or of the linearized plasmid DNAs for the qPCR standards (Table 1). PCR efficiencies of the standards were not significantly different from those of the samples (P > 0.05, Mann-Whitney *U* test). A standard curve was included in every PCR run. Consistency of technical replicates within each run was checked as a default operation by the StepOne software (standard deviations between replicates < 0.5). Consistency of data between different runs was assured by analyzing reproducibility of C_T values obtained from qPCR standards or randomly selected samples from any experimental condition (P > 0.05, Mann-Whitney *U* test). Consistency of parameters of standard curves belonging to different runs was also assessed (P > 0.05, Mann-Whitney *U* test).

2.5. Statistical analysis

Univariate comparisons of qPCR data from *in situ* baseline evaluations and heat stress experiments were performed using the non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney *U* test (P < 0.05), after deviations from parametric ANOVA assumptions were verified (Normality: Shapiro-Wilk's test; equal variance: Bartlett's test). Data analyses were performed using GraphPad Prism 6 software (GraphPad Inc.). Permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008) was performed on *in situ hsp70* baseline expressions. Log-transformed *hsp70* copy number variations were used to calculate similarity matrices based on the Euclidean distance (999 permutations). Factors considered were "growth mode" and "trophic strategy", that were used to calculate similarity matrices based on the

Euclidean distance (Anderson et al., 2008). An ordination analysis (carried out by Principal Coordinates, PCO) and data clustering using the PRIMER v6 software was performed on data from *in situ hsp70* baseline levels in the selected coral species and pattern of *hsp70* over-expressions following heat stress. Log-transformed *hsp70* levels under *in situ* baseline conditions and *hsp70* induction across the post heat-stress recovery period (expressed as the calculated AUC, Area Under the Curve of the *hsp70* transcriptional profiles for each species; Fig. S1 and Table S1, Supplemental material) were employed to calculate similarity matrices based on the Euclidean distance (999 permutations).

3. Results

3.1. Sequencing of hsp70 cDNAs

Using degenerated primers and a semi-nested PCR approach, sequences for partial hsp70 cDNAs from *B. europaea* (15), *A. calycularis* (17), *C. inornata* (11), and *C. caespitose* (9) were obtained. Positive clones included a partial ORF of about 450 bp corresponding to the central fragment of the hsp70 coding region. Representatives of HSP70 sequences have been submitted to the GenBank database (A. calycularis: MH644814; *B. europaea*: MH618777; *C. caespitosa*: MH644815; *C. inornata*: MH618776). The sequences were aligned together with a dataset of coral hsp70 nucleotide sequences retrieved from GenBank and the Reefgenomics.org databases [the complete dataset corresponds to that reported by Poli et al. (2017) for related protein sequences]. Sequences of hsp70 for the analyzed species shared a major sequence identity (about 62%) with hsp70s from members of the *Porites* family, while only an average 30% sequence identity with the available constitutive Scleractinian hsc70s. Amongst the selected Mediterranean

Table 2
PERMANOVA results on basal *hsp70* expression in the selected Mediterranean corals under field conditions (998 permutations).

Source	df	Pseudo-F	P(perm)
Growth mode	1	3.622	0.069
Trophic strategy	1	98.653	0.001
Growth mode x Trophic strategy	1	11.935	0.003

df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F.

corals, *B. europaea* and *A. calycularis* showed the highest pairwise sequence identity score (about 71%), while the lowest score (about 53%) was between *B. europaea* and *C. inornata*.

3.2. Between-species differences of in situ hsp70 baseline expression levels

Expression of *hsp70* transcript was assessed in *B. europaea*, *A. calycularis*, *C. inornata*, *C. caespitosa,* and *L. pruvoti* using an absolute quantitative real-time PCR assay (Fig. 4A). Results showed that the *hsp70* transcripts were expressed in all samples of each species. The zooxanthellate species *B. europaea* and *C. caespitosa* displayed significantly higher *in situ hsp70* levels compared to the azooxanthellae species. The only exception was represented by the azooxanthellate *C. inornata*, which showed *in situ hsp70* baseline levels similar to those of *B. europaea* (Fig. 4A). PERMANOVA analyses of *hsp70* expression revealed a significant interaction of the factors "trophic strategy" and "growth mode" (Table 2). Pairwise comparisons by permutation t-tests showed significantly higher *in situ hsp70* baseline expression levels of the colonial morphotype within zooxanthellate species (Fig. 4A), while no significant differences within azooxanthellate corals were found (Table 3).

3.3. Transcriptional responses and inter-species differences of the hsp70 expression profiles under a heat shock treatment

Changes of *hsp70* expression levels were evaluated following an acute heat shock (2 h at 32 °C) and different periods of post-stress recovery (0 h, 2 h, 24 h) at the control temperature (17 °C) (Fig. 3). For each species, values were normalized against mRNA expression levels at the onset of the experimental exposure to thermal stress (control samples, ctr), achieved after a 40-days acclimation period to laboratory conditions (Fig. S2, Supplemental material).

Among zooxanthellate species, *B. europaea* showed significantly increased hsp70 expression at 2 h post-stress recovery (Fig. 4B), with levels decreasing thereafter, while *C. caespitosa* showed no significant variation of *hsp70* levels (Fig. 4C). Amongst azooxanthellate species, *A. calycularis* showed the highest *hsp70* fold-change increase, with levels being significantly higher than controls from 0 h post-stress recovery and reaching a 3.5-fold and 3-fold up-regulation at 2 h and 24 h post-stress recovery, respectively (Fig. 4D). Levels of *hsp70* in *C. inornata* were significantly increased compared to controls at 0 h post-stress and recovered control values thereafter (Fig. 4E). *L. pruvoti* showed a small though significant *hsp70* over-expression at 2 h post-stress recovery,

Table 3
Permutation t-tests through PERMANOVA pairwise comparisons for the effects of the factor "growth mode" on basal *hsp70* expression amongst zooxanthellate or azooxanthellate species.

Pairwise comparisons (colonial vs solitary polyp)	t	P(perm)	Unique permutations
Azooxanthellate species	1.02	0.299	747
Zooxanthellate species	5.55	0.002	747

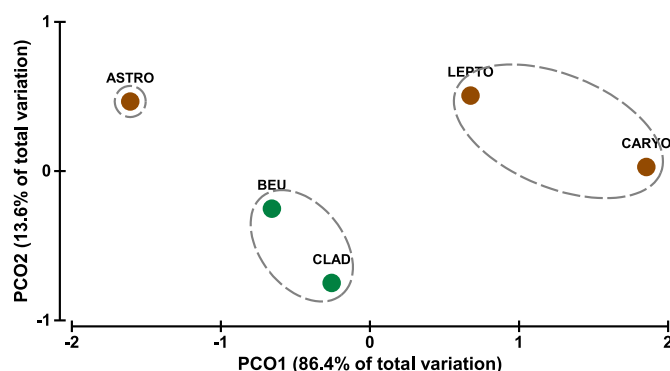t: t-Statistics; P(perm): probability of t.

Fig. 5. Principal coordinates ordination (PCO) bi-plot of the *hsp70* response with super-imposed cluster analysis (grey dotted lines) by condition (i.e., different morphotype and different symbiotic partnership) (Euclidean Distance resemblance matrix; 999 permutations). PCO and cluster analyses were performed on a resemblance matrix generated from both *in situ hsp70* baseline expression and *hsp70* induction across different periods of post heat-stress recovery (area under the curve, AUC, showing the *hsp70* fold change variations across the post-stress recovery period; Fig. S1 and Table. S1, Supplemental material). Green circles indicate the zooxanthellate species, orange circles indicate azooxanthellate species. ASTRO: *A. calycularis*; BEU: *B. europaea*; CARYO: *C. inornata*; CLAD: *C. caespitosa*; LEPTO: *L. pruvoti* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

and no significant variations at 0 and 24 h post-stress recovery (Fig. 4F).

A PCO analysis was applied to determine whether the factors "growth mode" and "trophic strategy" could discriminate samples by means of variations of *in situ hsp70* baseline expressions as well as of species-specific differences of *hsp70* response in the post heat-stress recovery experiment (Fig. 5). The latter parameter was measured by assessing the area under the curve describing *hsp70* transcriptional profiles across the recovery period post-stress (Fig. S1 and Table. S1, Supplemental material). Two principal coordinates (PCO1 and PCO2) were observed to explain 100% of total variance (86.4% and 13.6%, respectively). The super-imposed data clustering showed that samples from zooxanthellate *B. europaea* and *C. caespitosa* formed one group with marked separation from the azooxanthellate species (Fig. 4). Amongst the azooxanthellate corals, *A. calycularis* showed separation from *C. inornata* and *L. pruvoti*, likely due to the remarkably different *hsp70* transcriptional profile (i.e. higher AUC values) during the 24-h post stress recovery (Fig. 4).

4. Discussion

In this study, we explored whether five shallow-water coral species of the Mediterranean Sea with different growth modes (solitary *vs* colonial) and trophic strategies (zooxanthellate *vs* azooxanthellate) have differential transcriptional profiles of their *hsp70* expression *in situ* and in response to thermal stress. Notably, these species showed distinct susceptibilities towards temperature (Fig. 1).

Degenerated primers designed based on the multiple alignment of homologous hsp70 coral protein-coding nucleotide sequences allowed the identification of partial hsp70 sequences in *B. europaea*, *A. calycularis*, *C. inornata*, *C. caespitosa.* Sequence alignments and identity scores showed that these partial sequences encoded heat inducible HSP70s displaying a 60%–68% sequence identity with inducible HSP70s from *Porites australiensis*, while only about 30% sequence identity with the available constitutively expressed scleractinian hsc70s. These partial sequences were employed to design specific primer pairs to be used in absolute qPCR protocols. Validation by electrophoresis, cloning, and sequencing of qPCR products demonstrated that the same set of primers allowed *hsp70* transcriptional analysis in all the five Mediterranean coral species investigated in this study and detected no amplification of

algal symbiont *hsp70* transcripts. Consequently, these primers were used to assess *hsp70* expression across further Mediterranean coral species.

Transcript levels of *hsp70* were evaluated immediately after coral sampling to account for differences in mRNA expression under *in situ* conditions and to assess baseline expression levels in the natural environment. Further, *hsp70* expression was assessed in nubbins/polyps experimentally subjected to a short-term heat stress exposure (2 h at 32 °C) and after different periods of post-stress recovery (0–24 h at 17 °C). Coral species with higher *in situ hsp70* baseline levels had limited (*B. europaea*) or no evident (*C. caespitosa) hsp70* induction by heat stress. This is a well-known phenomenon, common to several invertebrates inhabiting shallow-water environments (Franzellitti and Fabbri, 2005; Tomanek, 2010), and has been termed "constitutive gene frontloading" (Barshis et al., 2013). The occurrence of a minimal constitutive *hsp70* expression is likely part of the strategy loaded by these organisms to cope with frequently encountered environmental stress conditions (Fabbri et al., 2008; Morris et al., 2013). This strategy is thought to confer physiological resilience by means of faster reaction at the protein level during transient heat stress (Bay and Palumbi, 2017; Bellantuono et al., 2012; Ruiz-jones and Palumbi, 2017). Previous studies highlighted a frontloading behavior of *hsp70* transcripts through reciprocal transplanting of *Acropora hyacintus* nubbins between sites experiencing distinct temperature regimes (Barshis et al., 2013; Bay and Palumbi, 2017), or assessing the influence of depth-related basal expression profiles on *hsp70* induction in *P. verrucosa* (Poli et al., 2017). In agreement with these observations, in this study the most remarkable differences in the *hsp70* responses were observed between zooxanthellate and azooxanthellate species, suggesting that symbiosis may play an important role in modulating stress response capabilities of the coral hosts. Specifically, zooxanthellate corals showed higher *in situ hsp70* baseline expression than the azooxanthellates, suggesting that this might be consequence of the symbiosis that is arguably highly susceptible to the effects of thermal stress. Furthermore, among zooxanthellate species, *C. caespitosa* showed higher *in situ hsp70* baseline levels than *B. europaea*, and remarkably different post heat-stress transcriptional profiles were observed between these two species. Although composition of *Symbiodinium* clades hosted by the two species was not assessed in this study, previous reports on the Tyrrhenian Sea showed that either *B. europaea* or *C. caespitosa* harbored clade B2, while clade A was found only in *B. europaea* (Barbrook et al., 2006; Meron et al., 2012). Physiologically, the "temperate" clade A is thought to confer potential resistance to short-term increases in temperature (Rodolfo-Metalpa et al., 2006a). Therefore, this finding may explain the more effective *hsp70* induction by heat stress displayed by *B. europaea*. Besides the putative influence of symbiosis, peculiar life history traits and adaptive features of these species may have a role in determining their observed *hsp70* responses. Being sympatric species, *B. europaea* and *C. caespitosa* have a widely overlapping habitat distribution; hence, differences with regard to different environmental exposure do not serve as an explanation for the observed species-specific responses. We may hypothesize a relationship between stress responsiveness at the transcriptional level and colonial (*C. caespitosa*) vs solitary (*B. europaea)* coral morphotype. More generally, coral morphology is thought to influence flow regimes and thermal fluxes at the tissue-water interface (Jimenez et al., 2011; Nakamura and Van Woesik, 2001), tissue thickness and availability of resources (Loya et al., 2001), and to affect diversity and light-absorbing properties of the zooxanthellae symbionts (Enríquez et al., 2005; Yost et al., 2013). A recent study modeled the relationships between allometric scaling and rates of heating/cooling in coral microenvironments as a result of physical and geometric constraints on body size and shape, and showed that, under similar water flow and irradiance conditions, smaller corals are subjected to comparatively higher stress levels than bigger corals (Ong et al., 2017). Consequently, during the heat treatments the (smaller) solitary morphotype (*B. europaea*) investigated in this study may have experienced relatively higher heat fluxes at the water-polyp interface compared to the (bigger) colonial *C. caespitosa*, thus showing higher *hsp70* induction.

Cladocora caespitosa has been subjected to recurring mass bleaching and mortality events due to anomalous heat waves in different areas of the Mediterranean Sea (Jimenez et al., 2016; Kersting et al., 2013; Rubio-Portillo et al., 2016). These events may have contributed to shaping physiological outputs of this species in response to temperature, likely diminishing the natural capacity of the species to recover (Kersting et al., 2013). The comparatively high(er) *in situ hsp70* baseline levels displayed by *C. caespitosa* may render this species less responsive to additional stress(ors). Indeed, no further *hsp70* up-regulation during post-stress recovery was observed, suggesting a limited ability of this species to further adjust its physiology to increasing temperatures. On the whole, both physiological (Rodolfo-Metalpa et al., 2006b, 2006a) and transcriptional (present study) outcomes support the finding of *C. caespitosa* living close to its upper thermal tolerance limit within the Mediterranean Sea (Rodolfo-Metalpa et al., 2006b). Considering that *C. caespitosa* is listed as endangered by the IUCN (Otero et al., 2017), these findings may contribute to the growing body of knowledge on the vulnerability of this species towards projected sea temperature rise.

Although *in situ hsp70* baseline expression was not significantly different amongst the azooxanthellate species, different temporal profiles were observed after heat shock exposure. Based on the magnitude of *hsp70* induction in heat-shock experiments, the following ranking of heat sensitivity can be defined: *A. calycularis* > *C. inornata* > *L. pruvoti*. This ranking agrees with the relative stress tolerances of the species predicted from variations of growth, reproduction, and population dynamics across environmental gradients (Caroselli et al., 2017, 2016a; 2016b) (Fig. 1). *Leptosammia pruvoti* appears the less sensitive amongst the azooxanthellate species investigated in this study, showing a very limited increase of *hsp70* expression only at 2 h post-stress recovery. Indeed, this species showed unaffected physiological outputs along extended latitudinal thermal and solar irradiance gradients (Airi et al., 2017; Caroselli et al., 2012a, 2011; Goffredo et al., 2007). *A. calycularis* resulted the most sensitive azooxanthellate species, showing the highest *hsp70* fold changes, in agreement with the relevant physiological effects of hypertermic stress observed in this species (Movilla et al., 2016; Prada et al., 2017). Compared to *L. pruvoti*, *A. calycularis* seems particularly sensitive to high summer temperatures, displaying a relevant increase in tissue mortality that probably made the corals more susceptible to the detrimental effects of further stress events (Prada et al., 2017). Notably, *A. calycularis* maintained significant *hsp70* up-regulation up to 24 h post-stress recovery, which may suggest a slow reactivity or a partial temperature-dependent impairment of the cellular machinery at the basis of its heat stress response, as previously observed in the temperature-sensitive oyster *Ostrea edulis* exposed to near-lethal temperature increases under a similar exposure scheme (Piano et al., 2004).

5. Conclusions

Data reported in this study indicate that transcriptional responses of the hsp70 gene align with previously reported relative differences in stress susceptibility of the coral species investigated. Although variability in environment requirements is considered a pivotal factor in determining the degree of stress resilience in shallow-water species, our results suggest that trophic strategy and morphology may play an important role in shaping coral resilience and physiological plasticity, as previously hypothesized (Samorì et al., 2017; Seveso et al., 2018). Combining baseline *hsp70* expression and heat stress induction allowed discriminating the potential of stress responses between zooxanthellate and azooxanthellate species, as well as, within the different trophic strategies, between colonial vs solitary corals. Whether scientific evidences are calling into question the role of symbiosis as a buffer towards the adverse effects of environmental stressors (Ellison et al., 2017; Levin et al., 2016; Schwarz et al., 2008), the relevance of coral

morphology in shaping holobiont response to environment variability is underestimated. In this light, results of this study let argue that bigger (colonial) morphotype may represent a winning physiological trait to promote stress tolerance and adaptation in a global warming scenario. Future studies incorporating further species should assess whether *hsp70* expression may serve as a hallmark of coral trophic status and morphological constraints. Understanding these molecular processes can contribute to disclose the relevant physiological traits underpinning these coral tolerances to stress, and this will help us predicting the future of shallow-water corals under projected scenarios of anthropogenic global change.

Competing interests

No competing interests declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.marenvres.2018.07.006.

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